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The short-term effectiveness of non-surgical treatment in reducing protease activity in gingival crevicular fluid from chronic periodontitis patients

C. M. S. Figueredo[1,2], A. Areas[2], L. A. Miranda[1,2], R. G. Fischer[2] and A. Gustafsson[1]

[1]Institute of Odontology, Division of Periodontology, Karolinska Institutet, Stockholm, Sweden; [2]Faculty of Odontology, Department of Periodontology, Rio de Janeiro State University, Rio de Janeiro, Brazil

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Abstract

Objectives: The aim of this study was to evaluate the short-term effect of non-surgical periodontal treatment on protease activity in gingival crevicular fluid (GCF) of patients with chronic periodontitis.

Material and Methods: After clinical examination, in which pocket probing depth, probing attachment level, plaque and bleeding indices were recorded, gingival fluid samples from 21 chronic periodontitis patients were collected from gingivitis (GP) and periodontitis (PP) sites with an intracrevicular washing method. Samples were taken in the same way from a group of patients with gingivitis alone (GG). The periodontitis patients received non-surgical periodontal treatment and were re-evaluated 30 days later. We compared elastase and collagenase activities before and after treatment. The former activity was measured with a low-weight substrate (S-2484) and inhibited by α-1-antitrypsin. Matrix-metalloproteinase-8 (MMP-8) was measured with an ELISA and collagenolytic activity with fluorescein-conjugated collagen type I as substrate.

Results: All clinical parameters showed a significant improvement after treatment ($p<0.05$) which was accompanied by a significant reduction in the values of total elastase activity, free elastase, MPP-8 and collagenolytic activity in both GP and PP sites ($p<0.05$). However, the latter sites continued to have higher levels of MMP-8 and collagenolytic activity than the former ones after treatment. The free elastase activity and the proportion of free elastase in GP and PP samples after treatment remained higher than in untreated GG samples.

Conclusion: This study shows that the clinical improvements after non-surgical treatment are accompanied by reductions in protease and neutrophil activities.

Key words: elastase; MMP-8; periodontal disease; protease activity

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Activity and inhibition of endogenous proteases, such as elastase and matrix-metalloproteinase-8 (MMP-8), are essential for the progression of periodontitis. Several studies have shown increases in the amounts and activity of these proteases, both mainly derived from neutrophils, in gingival crevicular fluid (GCF) from sites with periodontitis (Overall et al. 1987, Gustafsson et al. 1994, Ingman et al. 1996, Meyer et al. 1997, Figueredo & Gustafsson 1998a). It is not only the amounts of these proteases that differ, but also, and perhaps more importantly, the activity. In a previous study, our group has shown that more elastase remains active in GCF from sites with deep pockets and greater attachment loss (Figueredo & Gustafsson 1998b). Neutrophil elastase is a serine protease, which degrades elastin and several other functionally and structurally important proteins in the periodontium, including collagen,

proteoglycans and basement membrane components (Janoff 1985). Some investigators have evaluated the effects of treatment on the presence and activity of elastase (Buchmann et al. 2002a, b, Jin et al. 2002). However, the methods used in these studies must be considered. Substrates with low molecular weight are hydrolysed by free elastase and elastase bound to α-2-macroglobulin (E-A2MG) (Travis & Salvesen 1983). This means that measurements of elastase with such substrates cannot show whether elastase is free or inhibited, which is necessary to determine the tissue destructive capacity. Herein, we distinguished free elastase from E-A2MG by adding an excess of α-1-antitrypsin (A1AT) to the samples. A1AT is a very effective inhibitor of elastase, but cannot inhibit the activity from the E-A2MG complex (Travis & Salvesen 1983). Another methodological consideration is that free elastase cannot be recovered from paper strips (Gustafsson 1996), which suggests that intracrevicular washing rather than paper strips should be used to avoid retention of free elastase in the paper strips.

Apart from elastase, the neutrophil MMP-8 plays a central role in the turnover and degradation of periodontal tissues, especially in the degradation of type I collagen. MMP-8 is chiefly found in neutrophils, but it is also expressed by other cells in the periodontitis-affected area (Kiili et al. 2002). Higher levels of MMP-8 have been found in GCF of periodontitis patients (Ingman et al. 1994, Lee et al. 1995, Ingman et al. 1996). It is probably the main type of collagenase in GCF from patients with periodontitis (Ingman et al. 1996, Tervahartiala et al. 2000) and it has been suggested to be suitable for monitoring periodontal conditions (Sorsa et al. 1999, Chen et al. 2000). Some data show that it is the activity rather than the amount of MMP-8 that differs between sites with periodontitis and those with gingivitis (Romanelli et al. 1999). In this study we measured both the amount of MMP-8 and the collagenolytic activity.

To inhibit disease progression successfully, periodontal treatment must reduce the protease activity in the local inflammatory lesion. Therefore, the aim of this study was to measure neutrophil protease activity before and after non-surgical treatment.

Material and Methods

Patients

The patient sample consisted of 9 men and 12 women (mean age 44.5 ± 6.1 years), who had at least six sites with a pocket depth of 5 mm or more. Six patients were smokers. For purposes of comparison, we added data from a group of patients with gingivitis alone (GG) to the tables and figure. The gingivitis controls comprised 22 untreated patients, mean age 47.5 years. Six were smokers. All participants were volunteers with no ongoing general diseases or infections, gave their informed consent and the study protocol was approved by the Ethics Committees at Huddinge University Hospital, Huddinge, Sweden and Pedro Ernesto University Hospital, Rio de Janeiro, Brazil.

Sampling method and clinical recordings

Samples were taken from five to six deep pockets (probing pocket depth PPD $\geqslant 5$ mm) (PP – periodontitis sites in periodontitis patients) and 5–6 shallow ones (PPD < 5 mm) (GP – gingivitis sites in periodontitis patients). All of these sites were inflamed and had a GI > 2. The clinical examinations and the GCF samples were taken at baseline (day 0) and 30 days after treatment (day 30 AT). In the gingivitis group (GG), five to six sites were sampled in a similar manner.

GCF was collected with an intracrevicular washing device modified from Salonen & Paunio (1991). The sites to be sampled were isolated with cotton rolls and dried gently with an air syringe. Supragingival plaque was carefully removed before sampling. Each pocket selected was washed five times with 5 μl of PBS during continuous aspiration. The samples from the same type of site in each person were pooled together, diluted with PBS up to 1 ml and immediately centrifuged at $3000 \times g$ for 10 min. The supernatant was collected and frozen at -70°C, pending analysis.

We evaluated supragingival plaque by the plaque index (PI) of Silness & Löe (1964), gingival inflammation by the gingival index (GI) of Löe (1967) and measured the PPD and probing attachment level (PAL) after sampling.

Treatment

The patients with periodontitis received non-surgical periodontal treatment, which comprised instructions about oral hygiene and supra- and subgingival debridement (scaling and root planing) under local anaesthesia. The treatment took, on average, four sessions of 40 min, in 2 weeks. The scaling and root planing was performed with manual instruments (Gracey and McCall Curettes, Hirschfield Files, HU-Friedy® Mfg. Co. Inc., Chicago, IL, USA) and by a single trained operator. Re-evaluation was performed 30 days after completion of non-surgical treatment.

Chemicals and reagents

Samples and standards were diluted in phosphate-buffered saline (PBS), pH 7.4. The granulocyte elastase substrate S-2484 (L-pyroglutamyl-L-prolyl-L-valine-*p*-nitroaniline, mw 445.5 Da, Chromogenix AB, Mölndal, Sweden) was dissolved in dimethylsulphoxide to 8 mmol/l and the working solution was made up to 2 mmol/l by dilution in PBS. The alkaline phosphatase substrate, *p*-nitro-phenol phosphate (Janssen Chimica, Geel, Belgium), was diluted to 2.7 mmol/l in diethanolamine buffer, pH 10.0. The microtitre plates were washed with PBS + 0.05% polyoxyethylene sorbitan monolaurate (Tween® 20, Sigma Chemical, St Louis, MO, USA).

Elastase activity

One hundred μl of sample was mixed with 67 μl of substrate on a 96-well microtitre plate (Nunc Maxisorp, Nunc, Roskilde, Denmark). The mixture was incubated at $+37^{\circ}$C and the absorbency at 405 nm was read after 2 h in a spectrophotometer (Millenia Kinetic Analyzer, Diagnostic Product Corporation, Los Angeles, CA, USA). The elastase activity was expressed as mAbs.

To inhibit the elastase activity, 10 μl of A1AT 0.1% was added to 90 μl of sample and incubated during agitation for 15 min at room temperature. After inhibition, the samples were tested against elastase activity, as described above. The elastase activity inhibited by A1AT was regarded as free elastase and the remaining activity as elastase inhibited by A2MG.

MMP-8

A human MMP-8 (total) kit (Quantikine®, R6D Systems Inc., Minneapolis, MN, USA) was used in accordance with the manufacturer's instructions. Briefly, a

monoclonal antibody specific for MMP-8 had been precoated onto a microplate. Samples diluted 20 times and a standard were pipetted into the wells and incubated at room temperature for 2 h. The plates were then washed and a monoclonal antibody against MMP-8 conjugated to horseradish peroxidase was added and incubated again as before. After a new washing procedure, the substrate solution was added and the reaction stopped after 15 min with a stop solution. The absorbency at 450 nm was read within 20 min. in a spectrophotometer (Millenia Kinetic Analyzer, Diagnostic Product Corporation, Los Angeles, CA, USA).

Collagenolytic activity

The collagenolytic activity was measured with fluorescein-conjugated collagen type I as substrate (EnzChek® Collagenase Assay Kit, Molecular Probes, Leiden, The Netherlands). The stock solution of the substrate was diluted to 2 ml with distilled water. Twenty-five microlitres of the diluted substrate was added to a 100 μl sample. This mixture was incubated for 30 min and the amount of fluorescence released measured at an excitation wavelength of 490 nm and emission of 530 nm. Collagenase activity was expressed in arbitrary units. The samples were analysed in a VICTOR 2 (Wallac, Turku, Finland).

Statistical analysis

The significance of the differences between days 0 and 30 AT, as well as between G and P sites, was calculated with the Wilcoxon signed-rank test. The significance of the differences between gingivitis and periodontitis patients was calculated with the Mann–Whitney *U*-test. Probability values higher than 0.05 were considered as not significant. The correlations were calculated with the Spearman rank coefficient.

Results

The clinical findings are shown in Table 1. We found significant reductions after treatment in PI, GI and PPD in the GP and PP sites. In the latter, the mean pocket reduction was 1.9 mm ($p = 0.0002$). There were strong and significant correlations when the values for reduction in pocket depth were compared with those at baseline ($r = 0.8$, $p < 0.00001$) and with GI at baseline ($r = 0.6$, $p = 0.0001$).

Significant reductions in all values occurred on day 30 AT as compared to day 0, apart from the E-A2MG complex (Table 2, Fig. 1). The comparison between GP and PP samples at baseline (day 0) showed significantly higher levels of total elastase activity, free elastase and collagenolytic activity in the PP samples. On day 30 after treatment, the elastase values in GP sites were similar to those in PP sites, apart from E-A2MG. However, the values of MMP-8 and collagenolytic activity continued to be significantly higher in the PP sites

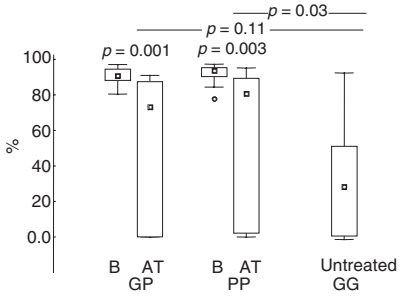

Fig. 1. Percentage of free elastase activity – percentage the total elastase activity that could be inhibited by α-1-antitrypsin – in GCF samples from gingivitis (GP) and periodontitis (PP) sites in 21 patients with periodontitis at baseline (B) and after treatment (AT). GG indicates samples from 22 untreated patients with gingivitis alone. The statistical significance of differences between baseline and after treatment were calculated with the Wilcoxon signed-rank test and those between GP/PP and GG were calculated with the Mann–Whitney *U*-test. Box plots indicate median, 10th, 25th, 75th and 90th percentiles.

Table 1. Comparison between mean values (SD) of plaque index (PI), gingival index (GI) and probing pocket depth (PPD) in gingivitis (GP) and periodontitis sites (PP) in 21 patients with periodontitis; data in 22 untreated patients with gingivitis alone (GG) are also shown

	GP day 0	*p*1	GP day 30	*p*2	PP day 0	*p*3	PP day 30	*p*4	GG	*p*5
PI	1.1 (0.6)	0.0006	0.5 (0.5)	NS	1.4 (0.5)	0.003	1.0 (0.5)	0.005	1.7 (0.6)	0.01
GI	1.4 (0.4)	0.0002	0.9 (0.5)	0.002	1.9 (0.3)	0.0001	1.2 (0.4)	0.03	1.3 (0.4)	NS
PPD (mm)	2.5 (0.5)	0.004	2.1 (0.5)	<0.0001	6.5 (0.8)	0.0002	5.2 (0.9)	<0.0001	2.2 (0.5)	NS

The significance of the differences was calculated with the Wilcoxon signed-rank test. *p*1 comparisons between GP on day 0 and day 30; *p*2 between GP on day 0 and PP on day 0; *p*3 between PP on day 0 and day 30; *p*4 between GP on day 30 and PP on day 30, *p*5 between GP on day 30 and GG (calculated with the Mann–Whitney *U*-test). Day 0 corresponds to baseline, and day 30 to the re-evaluation 30 days after treatment.
NS not significant.

Table 2. Mean values (SD) of the amounts of elastase activity (E activity), free elastase activity and elastase bound to α-2-macroglobulin (E-A2MG), all expressed as milliabsorbance (mAbs) and collagenolytic activity, expressed as arbitrary units (AU) and matrix metalloproteinase-8 (MMP-8), expressed in ng/ml, in 21 patients with periodontitis, according to gingivitis sites (GP) and periodontitis sites (PP); data on elastase activity in 22 untreated patients with gingivitis alone (GG) are also shown

	GP day 0	*p*1	GP day 30 AT	*P*2	PP day 0	*p*3	PP day 30 AT	*p*4	GG	*p*5
E activity	1419 (911)	0.001	529 (489)	0.02	2090 (902)	0.002	910 (762)	NS	528 (353)	NS
Free elastase	1303 (901)	0.009	415 (469)	0.04	1939 (899)	0.001	729 (737)	NS	321 (167)	NS
E-A2MG	117 (43)	NS	114 (29)	NS	151 (117)	NS	146 (63)	0.02	238 (307)	<0.001
MMP-8*	3.5 (3.0)	0.02	1.3 (1.0)	NS	7.8 (6.3)	0.007	2.6 (2.1)	0.02		
Collagenolytic activity	57145 (39106)	0.006	23530 (23765)	0.03	90654 (50915)	0.002	43239 (36996)	0.03		

The significance of the differences was calculated with the Wilcoxon signed-rank test. *p*1 comparisons between GP on day 0 and day 30; *p*2 between GP on day 0 and PP on day 0; *p*3 between PP on day 0 and day 30; *p*4 between GP on day 30 and PP on day 30. NS, not significant.
*$n = 14$ for MMP-8, *p*5 between GP on day 30 and GG (calculated with the Mann–Whitney *U*-test). Day 0 corresponds to baseline and day 30 to the re-evaluation 30 days after treatment.

than in the GP sites 30 days after treatment (Table 2). In comparison to GG samples, the free elastase activity and the percentage of free elastase remained higher in both GP and PP samples although the differences did not reach statistical significance (Table 2 and Fig. 1). E-A2MG was higher in the GG samples (Table 2).

We found no correlations between the results of clinical treatment and proteolytic activity at baseline or after treatment (data not shown).

Discussion

This study showed that 66% of the elastase activity, 59% of the free elastase and 67% of the collagenolytic activity was reduced by non-surgical periodontal therapy. This reduction was followed by a significant decrease in PPD and clinical increase in attachment levels, when the GP and PP sites were analysed together.

In general, all clinical data improved after therapy, which showed that non-surgical therapy was effective even in severe cases of chronic periodontitis. Other authors have also noted that such treatment is very effective in reducing pocket depth (Badersten et al. 1984, 1985). However, attachment loss after treatment and during maintenance may occur in the absence of signs of chronic inflammatory periodontal disease (for a review, see Egelberg 1999). We found that some sites continue to show collagenolytic activity 30 days after treatment and that there was no correlation between the clinical outcome and protease levels at baseline or after treatment. Previous studies have also shown that there is no correlation between clinical signs of inflammation and pro-inflammatory markers of inflammation (Wilton et al. 1992, Figueredo et al. 1999). Palcanis et al. (1992) found that the GI and PI did not vary significantly with the elastase levels and that the GCF elastase levels were significantly higher in sites showing increases in attachment and bone loss, which might predict for periodontal breakdown. We found that sites with tissue destruction had significantly higher elastase activity at baseline than those without such destruction, but the values were similar after treatment. The values of total elastase, free elastase and percentage of free elastase fell significantly 30 days after treatment, which confirms that, in general, non-surgical periodontal treatment is effective in reducing these biomarkers of inflammation. Our findings accord with other studies which have evaluated the levels of elastase and MMP-8 a brief period after non-surgical treatment (Chen et al. 2000, Jin et al. 2002). In patients with chronic and aggressive periodontitis, significant decrease has also been observed in the total GCF amount of elastase-α-1-proteinase inhibitor complex (EIC) 6 months after surgical therapy in patients with chronic and aggressive periodontitis has also been observed (Buchmann et al. 2002a). At baseline, the levels of EIC were higher in aggressive than in chronic periodontitis, but after treatment, this difference disappeared. Although EIC has been considered as a marker of disease activity in some conditions, such as rheumatoid arthritis (Momohara et al. 1997), it should be noted that this is a functionally inactive complex. Buchmann et al. (2002b) have also shown that a downregulation of crevicular neutrophil activity can be maintained for a long time.

In this study non-surgical treatment reduced the free elastase activity and the percentage of free elastase, but higher values tended to persist in gingivitis and periodontitis sites than in GCF samples from untreated patients with gingivitis alone albeit, less clinical inflammation. This could be interpreted as an indication that patients with periodontitis must maintain a very low degree of inflammation, that is a very good plaque control, which prevents an imbalance between proteases and antiproteases and subsequent progression of disease.

We also evaluated smokers and non-smokers (data not shown), but found no significant differences in the levels of elastase, which is in agreement with the study of Persson et al. (1999).

In conclusion, our results show that elastase levels in GCF, including total elastase activity, free elastase and percentage of free elastase, were significantly reduced 30 days after non-surgical periodontal treatment, as also was collagenolytic activity. However, PP sites continued to have higher levels of MMP-8 and collagenolytic activity than GP sites after treatment.

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Address:
Anders Gustafsson
Institute of Odontology
Karolinska Institutet
P O Box 4064
S-141 04 Huddinge
Sweden
Fax: +46 8 7118343
E-mail: Anders.Gustafsson@ofa.ki.se